Introduction:

The world's energy demand is threatened by the continued use of fossil fuels and concern over global warming (Yu *et al.,* 2003; Demirbas *et al.,* 2004). Fossil fuel combustion is responsible for 73% of the CO_2 production (Wildenborg *et al.,* 2005). The global warming issue has increased the awareness and interest in the development of methods to reduce green house gases emission (Lombardi *et al.,* 2003). The advanced technologies aims to focus on (i) reduced energy consumption (ii) increase the efficiency of energy conversion or utilization (iii) lower carbon content fuels (iv) enhanced natural sinks for CO_2 (v) capture and store CO_2. Reduced use of fossil fuels would reduce the amount of CO_2 produced, as well as reduce the levels of pollutants (Demirbas *et al.,* 2006[a]). Concern about global warming grows the search for energy sources that are renewable (Oliveria *et al.,* 2005).

There is a growing interest for ecological sustainable bio-fuels all over the world. A potential alternative to petroleum derived transportation fuels is biofuel (Govindaswamy and Vane 2007). Biofuels are the most promising renewable resources with a greater potential of environmental friendly nature. Biofuel has become a viable alternative to fossil fuels (Hsu *et al.,* 2011). Biofuels are renewable bio-based resource and liberates less CO_2 on combustion and reduces particulate emissions. Biofuel unlike, petroleum is renewable resource because of its inherent capacity to reappear or replenish themselves by quick recycling. Fuels that countries make from their own herbal and animal resources are briefly called biofuels. The conversion of feedstocks into biofuels exploits the renewable sources and reduces the polluting gases. Biofuels are both gaseous (biohydrogen and biomethane) and liquid (bioethanol, biodiesel, biomethanol, and biobutanol).

Alternative fuels such as biogas, bioethanol, biomethanol and biodiesel are in range but the most commonly used in bioethanol (Aydin and Ogut, 2014) which is (ethyl alcohol, grain alcohol, CH_3CH_2OH or ETOH) is a liquid biofuel produced from conversion of biomass by microorganisms. Bioethanol feedstock can contain either sucrose (e.g. sugarcane, sugar beet) or starch (e.g. corn, wheat) or be a lignocellulosic material (e.g. sugarcane bagasse, wood and straw) (Dias *et al.,* 2010). Bioethanol is generally made from feedstocks that are easily and cheaply available. Large amount of renewable biomass are available for conversion to liquid fuel ethanol (Balasubramanian *et al.,* 2011). First generation feedstock refers to those sources that serve as the nutrients for human and animals, namely sugar crops, cereal starches Second generation feedstock includes the non-edible and non-food biomass such as stalks of corn, grass, wood chips, old paper, bagasse, municipal solid waste,

agricultural residues. Third generation feedstock includes the microalgae and macroalgae seaweeds etc. These feedstocks are used for the production of bioethanol. The bioethanol production is among one of the oldest technology as it relies upon the fermentation of cereal grains, molasses or other materials rich in starch and sugar contents. The major steps in the biomass conversion are:

(a) Conversion of biomass and formation of fermentable sugars,

(b) Fermentation of sugars to bioethanol by the microorganisms,

(c) Separation and purification of the ethanol, usually by distillation to yield a fuel grade ethanol and by products that are used to produce other chemicals, heat or electricity.

No other sustainable option for production of transportation fuels can match ethanol made from lignocelluloses biomass with respect to its dramatic environmental, economic and infrastructure advantages (Pimentel and Patzek, 2005). Production of ethanol from lignocellulosic biomass seems very attractive and sustainable due to the renewable and ubiquitous nature of biomass and its non competitiveness with food crops. Lignocellulosic polymers are the most abundant raw materials that accounts for 90% of dry weight of plants (Jarboe *et al.,* 2007). Lignocellulosic materials have complexes of cellulose (20-50%), hemicelluloses (20-40%) and lignin (15-20%). Crop residues like cane bagasse, corn stover, wheat straw, rice hull, barley, hard wood, soft wood, newsprints, office waste, garden waste, municipal solid waste (Sanchez and Cardona, 2008). Production of bioethanol from lignocellulosic biomass includes delignification to release cellulose and complexes from lignin, conversion of carbohydrate polymers to produce simple and free sugars and fermentation of mixed hexoses and pentose sugars to produce ethanol. Because lignocellulosic materials are difficult to degrade it is necessary to analyze the changes in composition of biomass during the conversion (Lee, 1997). The utilization of lignocellulosic biomass for ethanol production necessitates the large-scale production technology to be cost effective and environmentally sustainable.

In the present study, *Shorea robusta* (Sal) seeds will be taken as a feedstock for bioethanol production. *Shorea robusta* (Sal) belongs to the family Dipterocarpaceae is a lignocellulosic substrate. It has an important role in economy in Jharkhand, Bihar, Orissa, Chhattisgarh and Madhya Pradesh. Sal seeds contain fat, triglycerides and serve as an ingredient for products like vanaspati, oil, soap, cocoabutter. The chemical composition of Sal seeds is 62% carbohydrates, 8% protein, 14.8% oil, 1.4% fiber, 2.3% ash and 10.8% water. Because of the availability and the high amount of carbohydrate content *Shorea*

robusta can be used as a source for bioethanol production. The major challenge met with the lignocellulosic material is their complexity which can be reduced by pretreatment methods.

A brief review of the work already done in the field:

Different researchers have worked on bioethanol production using various substrates. Toivala *et al.* (1984) studied about the alcoholic fermentation of D-xylose by yeast. Kida *et al.* (1989) studied the effect of aeration on the stability of continuous ethanol fermentation by yeast *Saccharomyces cerevisiae* H2A. Delgenes *et al.* (1996) worked on effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis,* and *Candida shehatae.* Olsson and Higerdal (1996) studied fermentation of lignocellulosic hydrolysate for ethanol production with different fermentation organisms among bacteria, yeasts, and fungi (natural as well as recombinant). Lee (1997) studied about the biological conversion of lignocellulosic biomass to ethanol and employing the delignification to convert cellulose and hemicelluloses to simple sugars, depolymerization of the carbohydrate polymers (cellulose and hemicelluloses) to produce free sugars, and fermentation of mixed hexoses and pentose sugars to produce ethanol. Wyman and Hinman (1999) emphasized on the technical progress, opportunities, and commercial challenges for biomass ethanol production with a better understanding of the technology to reduce the cost of economy.

Sommer *et al.* (2004) worked on the potential for using thermophilic anaerobic bacteria for bioethanol production from hemicelluloses and tested number of thermophilic enrichment cultures and new isolates of thermophilic anaerobic bacterial strains for their ethanol production from d-xylose growing optimally at 70–80 °C. Szambelan *et al.* (2004) studied about the use of *Zymomonas mobilis* and *Saccharomyces cerevisiae* mixed with *Kluyveromyces fragilis* for improved ethanol production from Jerusalem artichoke tubers which resulted in 2–12% yield for mixed cultures as compared to single microorganism. Demirbas *et al.* (2005) studied bioethanol from cellulosic materials a renewable motor fuel from biomass and concluded that the pretreatment methods efficiently convert the size and complexity of cellulosic biomass into fermentable sugars. Pimentel and Patzek (2005) studied ethanol production using corn, switch grass, and wood biodiesel production using soybean and sunflower and found physical and chemical factors limit the production of liquid fuels such as ethanol and biodiesel using plant biomass materials. Demirbas (2007 [b]) studied producing and using bioethanol as an automotive fuel and found the complex polymers can

be broken down by the pretreatment process which reduces the size and complexity by opening the structure for the cellulosic component which further gets hydrolysed by acids or enzymes into simpler sugars that gets fermented into ethanol. Lopez *et al.* (2007) worked on lignocellulose degrading enzymes produced by the ascomycete *Coniochaeta ligniaria* and related species: Application for a lignocellulosic substrate treatment and concluded that *Coniochaeta* and some related species constitute a newsource of cellulase, xylanase, lignin peroxidase and manganese peroxidase. Panesar *et al.* (2007) made a comparison of ethanol and temperature tolerance of *Zymomonas mobilis* strain in glucose and molasses medium. Sootsuwan *et al.* (2007) studied about the thermotolerant *Zymomonas mobilis* with comparison of ethanol fermentation capability with that of an efficient type strain TISTR 405 and found that at 39 ^{o}C ethanol was produced efficiently as that at 30 ^{o}C. Balata *et al.* (2008) studied about progress in bioethanol processing, feedstocks, pretreatment, hydrolysis, and economy. Choi *et al.* (2008) studied about ethanol production by *Zymomonas mobilis* CHZ2501 from industrial starch feedstocks. Elena (2009) about the bioethanol production from molasses by different strains of *Saccharomyces cerevisiae* due to their ability to hydrolyze sucrose from cane molasses into glucose and fructose. Marina *et al.* (2009) studied about the production of bioethanol and other bio-based materials from sugarcane bagasse with integration to conventional bioethanol production process.

Ali *et al.* (2011) studied ethanol fuel production through microbial extracellular enzymatic hydrolysis and fermentation from renewable agro based cellulosic wastes and employed cellulase producing *Aspergillus niger* and ethanol yielding *Saccharomyces cerevisiae* for the simultaneous saccharification fermentation and made a comparative study on stationary and shaking conditions. Arumugam and Manikandan (2011) studied about the fermentation of pretreated hydrolysate of banana and mango fruit wastes for ethanol production and revealed that the dilute acid pretreatment followed by the enzymatic saccharification gives production with optimized conditions. Gnanambal *et al.* (2012) studied biological pretreatment of six lignocellulosic wastes for bioethanol production. Modugu (2013) studied about the fermentative production of ethanol fuel from domestic waste by *Pichia stipitis* using garbage and kitchen as substrate using the acid hydrolysis to obtain fermentable sugars followed by the production of ethanol by the organisms. Aydin and Ogut (2014) studied determination of properties of ethanol-biodiesel fuels and produced biodiesel from safflower seeds to oil by means of raw oil extraction with heating at 90 ^{o}C.

Objectives of the work:

a. Collection of sample *Shorea robusta* (Sal) seeds
b. Isolation of bacterial strains from *Shorea robusta* (Sal) seeds during fermentation process
c. Screening of highest efficient bioethanol producing bacteria
    - Qualitative estimation of bioethanol
    - Quantitative estimation of bioethanol
    - Selection of bioethanol production bacterial strain
d. Characterization of isolated bacterial strains
    - Morphological characterization
    - Biochemical characterization
e. Identification and molecular characterization of highest bioethanol producing bacteria
f. Techniques developed for bioethanol production process
    - Pretreatment of the substrate for enhanced bioethanol production process
    - Standardization and optimization of parameters for bioethanol production

Noteworthy contribution in the field of proposed work:

Bioethanol is produced from various substrates which contain carbohydrates. Lignocelluloses are one of the most important renewable resource and several different works have been carried out using lignocellulosic substrates for the production of bioethanol. Zheng *et al.* (2009) worked on the biomass pretreatment for the cellulosic ethanol production and performed physical, chemical and biological pretreatment. Borah and Mishra (2011) studied production of biofuel from fruit waste with the objective for a relatively cheaper source for the production of ethanol. Sarkar *et al.* (2012) studied bioethanol production from agricultural wastes and used lignocellulosic substances as attractive feedstocks for bioethanol production because of the cost effectiveness, renewable and abundance. They stated that total delignification of lignocellulosic biomass is necessary to increase concentrations of fermentable sugars after enzymatic saccharification. Gnanambal *et al.* (2012) studied biological pretreatment of six lignocellulosic wastes for bioethanol production. They used agricultural wastes, sugarcane baggase, sweet sorghum, maize stover, groundnut shell, paddy straw and forestry waste, wood chips as source for bioethanol. *Trichoderma viride*

hydrolysed the pretreated substrates and *Saccharomyces cerevisiae* was used for the fermentation.

Akponah *et al.* (2013) studied bioethanol production from cassava effluent using *Zymomonas mobilis* and *Saccharomyces cerevisiae* isolated from rafia palm (*Elaesis guineesi*) SAP and results obtained on the fermentation activities of *Z. mobilis* and *S. cerevisiae* revealed that the amount of ethanol generated by *Z. mobilis* was more than that generated by *S. cerevisiae*. Beliya *et al.* (2013) also worked on deoiled rice bran for bioethanol production and studied different parameters for optimization. Tutt *et al.* (2014) studied steam explosion pretreatment method for bioethanol production from floodplain meadow hay and investigated the importance of pretreatment method at different temperatures on the conversion rates of sugar and bioethanol production efficiencies.

Proposed methodology:

1. **Collection of sample:-**

   The sample *Shorea robusta* (Sal) seeds will be collected from Village Sargipal, Bakawand range, Jagdalpur forest region, Chhattisgarh and will be stored in clean poly bags at room temperature.

2. **Isolation of bacterial strains from *Shorea robusta* (Sal) seeds during fermentation process:-**

   Bacterial strains will be isolated from *Shorea robusta* (Sal) seeds during fermentation process for bioethanol production

3. **Screening highest efficient bioethanol producing bacteria:-**

   Isolated bacterial strains will be screened for their fermentative property through carbohydrate fermentation test.

   a) Qualitative estimation of bioethanol:-

   Production of bioethanol will be confirmed by Jones reagent test (Bowden *et al.*, 1946) of screened bacterial strains.

   b) Quantitative estimation of bioethanol:-

   The amount of bioethanol will be estimated by Specific Gravity Method (Pharmacopoeia of India, 1985) of screened bacterial strains.

c) Selection of bioethanol production bacterial strain:-

The screened bacterial strains which will give the bioethanol production will be selected.

4. Characterization of isolated bacterial strains:-

Screened bacterial strains will be characterized on the basis of their morphology and biochemical tests.

5. Identification and molecular characterization of highest bioethanol producing bacteria:-

The highest bioethanol producing bacteria will be identified by molecular method (16s RNA sequencing) at species level.

6. Techniques developed for bioethanol production process:-

a. Pretreatment of the substrate for enhanced bioethanol production process:-

Pretreatment plays a key role in the overall efficiency of hydrolysis and fermentation step since the purpose of pretreatment is to remove structural ad compositional impediments to hydrolysis. The different pretreatment studied will be:

- Physical pretreatment
- Chemical pretreatment
- Biological pretreatment

b. Standardization and optimization of parameters for bioethanol production:-

Different parameters play an important role in process of fermentation and affect the bioethanol production. For this purpose these following parameters will be optimized:

- Static and stirred condition
- Temperature
- pH
- Nutrients

7. Analysis of data:-

Data will be statistically analyzed and interpreted by using desirable software.

Expected outcome of the work:

Decrease in fossil fuel reserves is due to the exponential worldwide energy need. This situation is alarming and needs to be solved and leads to the identification of alternative fuel sources (Gnanambal *et al.* 2012). The objective of present study is to produce bioethanol from lignocellulosic biomass, *Shorea robusta* (Sal) seeds and isolation and screening of bacterial strains for bioethanol production. Morphological and biochemical characterization of the screened microbes and molecular identification of highest efficient strain will describe the capability and features of bioethanol producing organisms. The different optimized parameters will enhance the capability of microbes to their optimum range that will result in increased efficiency in the conversion rates and bioethanol production. The pretreatment will improve further degradability of the substrate making the process more rapid and convenient. Production of bioethanol from *Shorea robusta* (Sal) seeds will explore the new lignocellulosic biomass for bioethanol production. More advancement and modifications will make the process feasible.

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Signature of Supervisor
(Prof. S. K. Jadhav)

Dr. S. K. JADHAV
Professor & Head
SOS in Biotechnology
Pt. Ravishankar Shukla University
RAIPUR-492 010 (C. G.)

Signature of Co- Supervisor
(Prof. K. L. Tiwari)

Dr. K. L. Tiwari
Former Prof. & Head
School of Studies in Biotechnology
Pt. Ravishankar Shukla University
RAIPUR-492 010 (C. G.)

Ankita

Signature of the Candidate
(Ankita Choudhary)

Forwarded

12/1/15

Chairman DRC

Dr. K. L. Tiwari
Former Prof. & Head
School of Studies in Biotechnology
Pt. Ravishankar Shukla University
RAIPUR-492 010 (C. G.)

Chairman (DRC)
SOS in Biotechnology
Pt. Ravishankar Shukla University
Raipur-492010 (C. G.)